PCT/GB 99/04268

The Patent Office 09/869696

INVESTOR IN PEOPLE

GB99/4268

# PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ



I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrew Craig

Dated 14 February 2000

Patents Form 1/77



The **Patent Office**

06SEP99 E474216-1 D02973
P01/7700 0.00 - 9920837.3

# Request for grant of a patent

**The Patent Office**
Cardiff Road
Newport
Gwent NP9 1RH

| | | |
|---|---|---|
| 1 | Your reference | MRH/P15464B |
| 2 | Patent application number | **9920837.3** |
| 3 | Full name, address and postcode of the applicant<br><br>Patents ADP number<br><br>State of incorporation | ML Laboratories Plc<br>17 Hanover Square<br>LONDON<br>W1R 9AJ<br>7115280005<br><br>United Kingdom |
| 4 | Title of the invention | GENE THERAPY |
| 5 | Name of agent<br>Address for service<br><br>Patents ADP number | Harrison Goddard Foote<br><br>Belmont House<br>20 Wood Lane<br>Headingley<br>Leeds<br>LS6 2AE |

| 6 | Priority applications | Country | Priority App No | Date of Filing |
|---|---|---|---|---|
| | | | | |

| | | | |
|---|---|---|---|
| 7 | Parent application (eg Divisional) | Earlier Application No | Date of Filing |
| | | | |
| 8 | Statement of Inventorship Needed? | No | |
| 9 | Number of sheets for any of the following (not counting copies of same document) | | |
| | Continuation sheets of this form | | |
| | Description | **12** | |
| | Claims | | |
| | Abstract | | |
| | Drawings | **2** +2 | |
| 10 | Number of other documents attached | | |
| | Priority documents | | |
| | Translations of priority documents | | |
| | P7/77 | | |
| | P9/77 | | |
| | P10/77 | | |
| | Other documents | | |
| 11 | I/We request the grant of a patent on the basis of this application. | | |
| | Signature Harrison Goddard Foote | Date 3 Sep 1999 | |
| 12 | Name and daytime telephone number of person to contact in the United Kingdom | Michael Harrison<br>+44 113 2258350 | |

# GENE THERAPY

## INTRODUCTION

This invention relates to gene therapy and to the invention described in our corresponding British patent application No 9900009.3 filed on 4 January 1999.

Gene directed enzyme prodrugtherapy (GDEPT) is one of a number of approaches currently being developed for the treatment of cancer. In this approach tumor cells are primed for increased susceptibility to a prodrug by first transfecting them with a gene encoding an enzyme capable of activating the prodrug. In this way, for example, 5-fluorocytosine can be activated by cytosine deaminase to produce the cytotoxic product 5-fluorouracil. Many other combinations of prodrug and activating enzyme have been described. [1] The treatment can be rendered specific to tumor cells by placing the gene encoding the activating enzyme under the control of a tumor-specific promoter, such as erbB-2. Thus, it is possible to limit expression of the activating enzyme to tumor cells.[2]

Produrgs for GDEPT have generally been based on conventional anti-cancer drugs, such as ifosfamide and 5-fluorocytosine, but often such compounds have limiting and serious side-effects. However, any prodrug that can be enzymatically converted to a potent cytotoxic agent could be used. It is desirable that the toxic metabolite should have a limited sphere of action so that it destroys the transformed cell and its immediate bystanders, but does not produce damage to adjacent normal tissue or systemic toxicity. In fact, the extent of the bystander effect of the cytotoxic product is an important consideration as tumor cells that fail to become transfected with the prodrug activating gene can be killed in this way. Thus, it may not be necessary to transfect all tumor cells to accomplish complete tumor cell killing. Acetaminophen, which is cytotoxic through the cytochrome P450-mediated generation of a chemically reactive metabolite, N-acetylbenzoquinoneimine (NABQI) may be a suitable prodrug for use in GDEPT.

Acetaminophen is a widely used and well tolerated analgesic in therapeutic doses. Overdosage in humans and certain susceptible species produces serious, often fatal, liver lesions.[3] Acetaminophen is largely eliminated in the liver by conjugations to form the sulphate and glucuronide derivatives. A minor pathway of metabolism is oxidation by the hepatic cytochrome P450 system to form NABQI, a chemically reactive, electrophilic species. This compound is normally detoxified by direct conjugation with reduced glutathione (GSH). However, in overdosage sufficient NABQI is formed to deplete hepatic GSH allowing the reactive metabolite to interact with tissue macromolecules leading to cell death.[4-6]

In human liver the P450 enzymes CYP1A2, CYP3A4 and CYP2E1 catalyse the formation of NABQI[7] but our preliminary studies suggest that CYP1A2 is the major form involved at concentrations of acetaminophen that causes NABQI-induced toxicity.[8] Therefore, the aim of this present study was to assess the use of acetaminophen and human CYP1A2 as prodrug and activating enzyme, the extent of the bystander effect of NABQI released from activiating cells and the susceptibility of a variety of tumor cell types to this cytotoxic agent.

## MATERIALS AND METHODS

*Cell Culture*

V79 MZ Chinese hamster cells were maintained in Dulbecco-Vogt's modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum 9FCS), 2 mM L-glutamine, penicillin (100 U/ml and streptomycin (100 μg/ml) (all tissue culture reagents were obtained from Gibco BRL, Paisley, UK). H1A2 MZ cells, which are V79 MZ cells transfected with the human *CYP1A2* gene,[9] were also maintained in supplemented DMEM with the addition of geneticin at a concentration of 4 mg/ml. SK-OV-3 cells were grown in DMEM supplemented with 15% FCS and 2 mM L-glutamine, without the addition of antibiotics. RPMI-1640 medium with 10% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) was required by the HCT116 cells. These cell lines were maintained at 37 °C with 100% humidity

and 5% $CO_2$. MDA-MB-361 cells were grown in Leibovitz (L-15) medium supplemented with 15% FCS and 2 mM L-glutamine maintained at 37°C with 100% humidity and did not require $CO_2$. At confluence cells were removed from tissue culture flasks by inubation with trypsin-EDTA for 5 min, diluted 1 : 3-1 : 6 in fresh medium and seeded onto fresh flasks. The tumor cell lines were obtained from the European Collection of Cell Cultures.

*H1A2 MZ cells*

The expression of human CYP1A2 in these cells was confirmed by measurement of 7-ethoxyresorufin O-deethylase and 7-methoxyresorufin O-deethylase activities on a cytosol-free protein fraction as described previously.[9] The respective values obtained of 5.4 ± 0.1 and 12.1 ± 0.2 pmol/min/mg protein (n=6) are similar to those reported previously, i.e. 6.5 and 12.8 pmol/min/mg protein[9] and no activity was detected in the parental V79 MZ cells. Further, the expression of human CYP1A2 in the cytosol-free protein fraction of H1A2 MZ cells, but not V79 MZ cells was also demonstrated by western blotting using an antibody specific for this P450 enzyme[10] (data not shown).

*Cell Viability*

For cell viability experiments 200,000 cells per well were plated on 12 well plates (Beckton-Dickenson, Oxford, UK), using the medium and conditions required by the tumor cell line and allowed to adhere overnight. After this the cells were washed with PBS before the addition of acetaminophen in 0.1 ml PBS (concentrations of 0.1 - 20 mM acetaminophben were dissolved in PBS by sonication) and then maintained at 37°C. At the appropriate time points cells were removed from the plates by trypsinisation and collected by centrifugation. Cell viability was measured as ability to exclude trypan blue. Cells were counted at x100 magnification using an Improved Neubauer haemocytometer; all cell counts are the mean of duplicate determinations of five fields from duplicate experiments.

## RESULTS

Incubating H1A2 MZ cells, which express CYP1A2, with a range of concentrations of acetaminophen (0.1-20 mM) for 6 h allowed the determination of cytotoxic concentrations (Fig. 1). No cytotoxicity was observed in the absence of acetaminophen or with 0.1 mM acetaminophen. However, after exposure to 1 mM acetaminophen viability fell to 62%. Incubation with 4 mM acetaminophen resulted in a further reduction in viability to 8%. Higher concentrations of acetaminophen resulted in a similar amount of cell killing (Fig. 1). In contrast, incubation of V79 MZ cells, which lack CYP1A2, with acetaminophen resulted in no loss of cell viability (Fig. 1). Therefore, as 4 mM acetaminophen was the minimum dose to give the maximum effect, this concentration was selected for further experiments.

To determine if the toxic metabolite produced by CYP1A2 had a cytotoxic effect on bystander cells incapable of activating acetaminophen, H1A2 MZ cells were mixed with V79 MZ cells before exposure to 4 mM acetaminophen. The percentage decline in total cell viability greatly exceeded the percentage of acetaminophen-activating cells in the mixture indicating a significant bystander effect (Fig. 2). In the presence of 5% H1A2 MZ cells the viability of the mixed cell population was reduced to 52%, and as the proportion of H1A2 MZ cells was increased the number of viable cells in the mixture declined (Fig. 2, Table 1) and a near maximal effect was found with a mixture of an equal number of V79 MZ and H1A2 MZ cells (Fig. 2). In contrast, no decline in cell viability was observed in V79 MZ cells incubated in 4 mM acetaminophen or H1A2 MZ cells incubated in phosphate-buffer saline (PBS) without acetaminophen (Fig. 2, Table 1).

The susceptibility of tumor-derived cells to cytotoxicity produced by the activation of acetaminophen was investigated by mixing H1A2 MZ cells with SK-OV-3, HCT116 and MDA-MB-361 cells and incubating them in the presence of 4 mM acetaminophen for 6 h. It was found that like V79 MZ cells, SK-OV-3 cells were highly sensitive as viability fell progressively as the prorportion of H1A2 MZ cells was increased (Table 1). HCT116 cells showed a disproportionate decrease in

viability when incubated with 5 and 10% H1A2 MZ cells, although with 50% H1A2 MZ cells the amount of cell killing could be accounted for by loss of H1A2 MZ cells alone (Table 1). However, MDA-MB-361 cells appeared to be highly resistant to cytotoxicity as the reduction in cell viability was similar to the proportion of H1A2 MZ cells present (Table 1).

To determine if the residual viable cells were programmed to die, as we have found previously,[11] attempts were made to culture the various cell types in normal growth medium after they had been exposed to acetaminophen for 6 h in the presence of the activating H1A2 MZ cells. It was found that after 24 h in culture, the viability of V79 MZ, SK-OV-3 and HCT116 cells mixed with as little as 5% H1A2 MZ cells fell to zero (Table 1). Only MDA-MB-361 cells showed resistance to cell killing, although even with these cells viability was reduced to 18% in cultures comprising an equal number of MDA-MB-361 and H1A2 MZ cells and no viable cells were found after 48 h (Table 1). Nevertheless, with fewer H1A2 MZ cells present viability increased with time, presumably as the number of dividing MDA-MB-361 cells increased during culture.

## DISCUSSION

Acetaminophen can be activated by oxidation catalysed by human CYP1A2 to form the cytotoxic compound NABQI. The combination of acetaminophen as prodrug and CYP1A2 as activating enzyme has a potential application in GDEPT. It has been demonstrated here that a sufficient amount of NABQI produced in cells transfected with human CYP1A2 to cause cytotoxicity. Further, enough NABQI is released from activating cells to cause cytotoxicity in neighbouring cells. Such a bystander effect was readily demonstrated in V79 MZ cells. However, human tumor-derived cells showed a range of sensitivities. An extensive bystander effect was found using SK-OV-3 cells, an ovarian tumor derived cell line, whereas, HCT-116 cells, which are derived from a colon tumor, appeared to be somewhat more resistant to the cytotoxic effect of NABQI when assessed immediately after a 6 h incubation period. However, both of these tumor cell types appeared to have been markedly damaged by exposure to NABQI as they failed to survive in culture. On the other hand, the MDA-

MB-361 cells, which originate from a breast tumor, displayed resistance of the ctyotoxic effect of NABQI, with only those cells exposed to the highest concentration being affected.

NABQI is capable of arylating and oxidising protein thiol groups, although studies with thiol reductants such as dithiothreitol[11] and N-acetylcysteine[12] suggested that the reversible oxidation of thiols (i.e. "oxidative stress") rather than arylation is responsible for cell death from acetaminophen.[6] Irrespective of the exact mechanism, the key factors that determine the toxicity of acetaminophen are the rate of production of NABQI as determined by the activity of the relevant cytochrome P450 enzymes and the starting level and extent of depletion of GSH. Thus rat liver is relatively resistant to acetaminophen, but not pre-formed NABQI, because the rate of formation of the reactive metabolite is insufficient, even at very high concentrations of acetaminophen, to deplete GSH.[13] In contrast, hamsters are very sensitive because acetaminophen is rapidly and extensively oxidised to NABQI. Human liver hepatocytes exhibit a range of sensitivities which correlate with the rate of oxidation of acetaminophen to NABQI.[13] Toxicity in overdosed individuals is confined to the liver which is the only organ that has the required level of enzyme activity to generate NABQI to deplete GSH, a prerequisite for cell damage and death. The hepato-toxicity of acetaminophen can be greatly increased in all species by prior depletion of GSH with chemicals such as diethylmaleate.[14] Thus the toxicity of acetaminophen is dependent upon the balance between the activity of the NABQI-generating enzyme and the concentration of GSH.

GSH concentrations in breast tumors (913nmol/g tissue) are two-fold greater than in normal breast tissue[15] but are less than 20% of those found in normal human liver (>5000 nmol/g tissue). Thus if tumor cells can be made to express NABQI-producing activity similar to human liver, therapeutic doses of acetaminophen should be selectively cytotoxic to the tumor cells. In addition, if used clinically in GDEPT, it should be possible to selectively protect the liver from any toxic effects of

acetaminophen by oral administration of GSH precursors such as methionine or N-acetylcysteine[16] that elevate GSH in the liver but not other tissues.[17]

It might be possible to increase the efficiency of the enzyme activating system by replacing human CYP1A2 with another P450 enzyme with a greater capacity for acetaminophen activation. Although human CYP2E1 and CYP3A4 are known to catalyse this reaction,[7] the rates relative to CYP1A2 at high concentrations of acetaminophen have yet to be determined. Alternatively, the orthologous rodent forms of CYP1A2, CYP2E1 or CYP3A4 may provide the source of a more efficient enzyme.

In conclusions, this study has demonstrated the potential of using acetaminophen as a prodrug when combined with a cytochrome P450-mediated activation system.

**REFERENCES**


1. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther.* 1995;2:702-709

2. Harris JD, Gutierrez AA, Hurst HC, Sikora K, Lemoine NR. Gene therapy for cancer using tumour-specific prodrug activation. *Gene Ther.* 1994;1:170-175

3. Davidson DG, Eastham WN. Acute liver necrosis following overdose of paracetamol. *Br Med J.* 1966;5512:-1990.

4. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther.* 1973;187: 211-217

5. Miner DJ, Kissinger PT. Evidence for the involvement of N-acetyl-p-quinoneimine in acetaminophen metabolism. *Biochem Pharmacol.* 1979;28: 3285-3290.

6. Boobis AR, Fawthrop DJ, Davies DS. Mechanisms of cell death. *Trents Pharmacol Sci.* 1989;10:275-280.

7. Patten CJ, Thomas PE, Guy RL, Lee M, Gonzalez FJ, Guengerich FP, Yang CS. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem Res Toxicol.* 1993;6:511-518.

8. Thatcher NJ, Murray S, Edwards RJ, Davies DS. Measurement of N-acetylbenzoquinoneimine formation by human hepatic microsomes. *12th International Symposium on microsomes and Drug Oxidations* 1998; abstract 74.

9. Wolfel C, Heinrich-Hirsch B, Schulz-Schalge T, Seidel A, et al. Genetically engineered V79 Chinese hamster cells for stable expression of human cytochrome P450IA2. *Eur J Pharmacol.* 1992;228:95-102

10. Edwards RJ, Murray BP, Singleton AM, Murray S, Davies DS, Boobis AR. Identification of the epitope of an anti-peptide antibody which binds to CYP1A2 in many species including man. *Biochem Pharmacol* 1993;46:213-220.

11. Tee LB, Boobis AR, Huggett AC, Davies DS, Reversal of acetaminophen toxicity in isolated hamster hepatocytes by dithiothreitol. *Toxicol Appl Pharmacol.* 1986;83:294-314.

12. Boobis AR, Tee LB, Hampden CE, Davies DS. Freshly isolated hepatocytes as a model for studying the toxicity of paracetamol. *Food Chem Toxicol.* 1986;24:731-736.

13. Tee LB, Davies DS, Seddon CE, Boobis AR. Species differences in the hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. *Biochem Pharmacol.* 1987;36: 1041-1052.

14. Potter WZ, Thorgeirsson SS, Jollow DJ, Mitchell JR. Acetaminophen-induced hepatic necrosis. V. Correlation of hepatic necrosis, covalent binding and glutathione depletion in hamsters. *Pharmacology.* 1974;12: 129-143.

15. Perry RR, Mazetta JA, Levin M, Barranco SC. Glutathione levels and variability in breast tumors and normal tissue. *Cancer.* 1993; 72: 783-787.

16. McLean AE, Day PA. The effect of diet on the toxicity of paracetamol and the safety of paracetamol-methionine mixtures. *Biochem Pharmacol.* 1975; 24: 37-42.

17. Aebi S, Lauterburg BH. Divergent effects of intravenous GSH and cysteine on renal and hepatic GSH. *Am J Physiol.* 1992; 263:348-352.

**Legend to Table 1.** Bystander effect on viability produced by incubating H1A2 MZ cells with various cell lines in the presence of paracetamol. Tumour cells or V79 MZ cells were co-cultured overnight with various mixtures of H1A2 MZ cells (as indicated in the Table), washed with PBS and incubated with 4 mM paracetamol in PBS for 6 h at 37°C. the cells were then washed once with PBS and maintained for either 24 or 48 h in culture medium appropriate to each cell type, as detailed in the Methods section. At 0, 6, 24 or 48 h the cells were washed in PBS and viability of the mixed cell population determined by trypan blue exclusion. The data shown are mean values ± SEM of 4 separate determinations. Statistical significance was

determined at each time point by comparing viability measurements in the mixed cell populations with both the viability determined at 0 h and in the absence of H1A2 MZ cells in the mixture 2 x 2 contingency tables using the 2-tailed Chi-squared test with Yate's correction. Viability was determined in a total of 1500 cells at each time point. Levels of significance are indicated as **$p<0.001$; ***$p<0.0001$.

Table 1.

| Cell type | H1A2 MZ cells (%) | Viability (%) 0 h | 6 h | 24 h | 48 h |
|---|---|---|---|---|---|
| V79 MZ | 0 | 99 ± 1 | 94 ± 7 | 98 ± 1 | - |
| | 5 | 98 ± 1 | 52 ± 5*** | 0 ± 0*** | - |
| | 10 | 98 ± 1 | 39 ± 6*** | 0 ± 0*** | - |
| | 25 | 97 ± 2 | 20 ± 4*** | 0 ± 0*** | - |
| | 50 | 96 ± 3 | 15 ± 7*** | 0 ± 0*** | - |
| SK-OV-3 | 0 | 97 ± 1 | 98 ± 2 | 96 ± 3 | - |
| | 5 | 98 ± 1 | 55 ± 2*** | 0 ± 0*** | - |
| | 10 | 98 ± 1 | 43 ± 7*** | 0 ± 0*** | - |
| | 25 | 97 ± 1 | 22 ± 11*** | 0 ± 0*** | - |
| | 50 | 98 ± 1 | 16 ± 9*** | 0 ± 0*** | - |
| HCT116 | 0 | 96 ± 1 | 96 ± 5 | 96 ± 2 | - |
| | 5 | 98 ± 1 | 69 ± 9*** | 0 ± 0*** | - |
| | 10 | 98 ± 1 | 60 ± 6*** | 0 ± 0*** | - |
| | 25 | 97 ± 1 | 56 ± 4*** | 0 ± 0*** | - |
| | 50 | 98 ± 1 | 42 ± 6*** | 0 ± 0*** | - |
| MDA-MB-361 | 0 | 96 ± 1 | 98 ± 4 | 95 ± 3 | 97 ± 1 |
| | 5 | 98 ± 2 | 85 ± 2** | 84 ± 7** | 92 ± 4 |
| | 10 | 96 ± 3 | 74 ± 7*** | 81 ± 9** | 94 ± 7 |
| | 25 | 97 ± 1 | 70 ± 11*** | 78 ± 9*** | 91 ± 6 |
| | 50 | 98 ± 3 | 42 ± 4*** | 18 ± 5*** | 0 ± 0*** |

## Figure Legends

**Figure 1.** The effect of paracetamol on the viability of V79 MZ and H1A2 MZ cells. V79 MZ cells (stipplied bars) and H1A2 MZ cells (filled bars) were allowed to adhere to the wells of tissue culture plates overnight, washed with PBS and then incubated with various concentrations of paracetamol for 6 h at 37 °C. Viability was determined by the ability of cells to exclude trypan blue. Data is represented as the mean ± SEM of 4 separate determinations and analysed by comparing the viability in H1A2 MZ cells with V79 MZ cells at each concentration of paracetamol using the 2-tailed unpaired Student's t-test. Levels of significance are indicated as *$p<0.01$; ***$p<0.0001$.

**Figure 2.** Bystander effect on viability produced by incubating paracetamol-activating H1A2 MZ cells with non-activating V79 MZ cells. V79 MZ cells were co-cultured overnight with various mixtures of H1A2 MZ cells, washed with PBS and then incubated with 4 mM paracetamol for up to 6 h at 37°C. The cells were then washed in PBS and the viability of the mixed cell population determined by trypan blue exclusion. The cultures comprised V79 MZ cells only (open squares), and V79 MZ cells mixed with 5% (solid squares), 10% (open triangles), 25% (open circles) and 50% (solid circles) H1A2 MZ cells. In addition, the viability of H1A2 MZ cells in the presence (crosses) and absence (solid triangles) of paracetamol is shown. Data is represented as the mean ± SEM of 4 separate determinations. Statistical analysis of the data at the 6 h time point is presented in Table 1

P15464B

Fig 1

(6k)

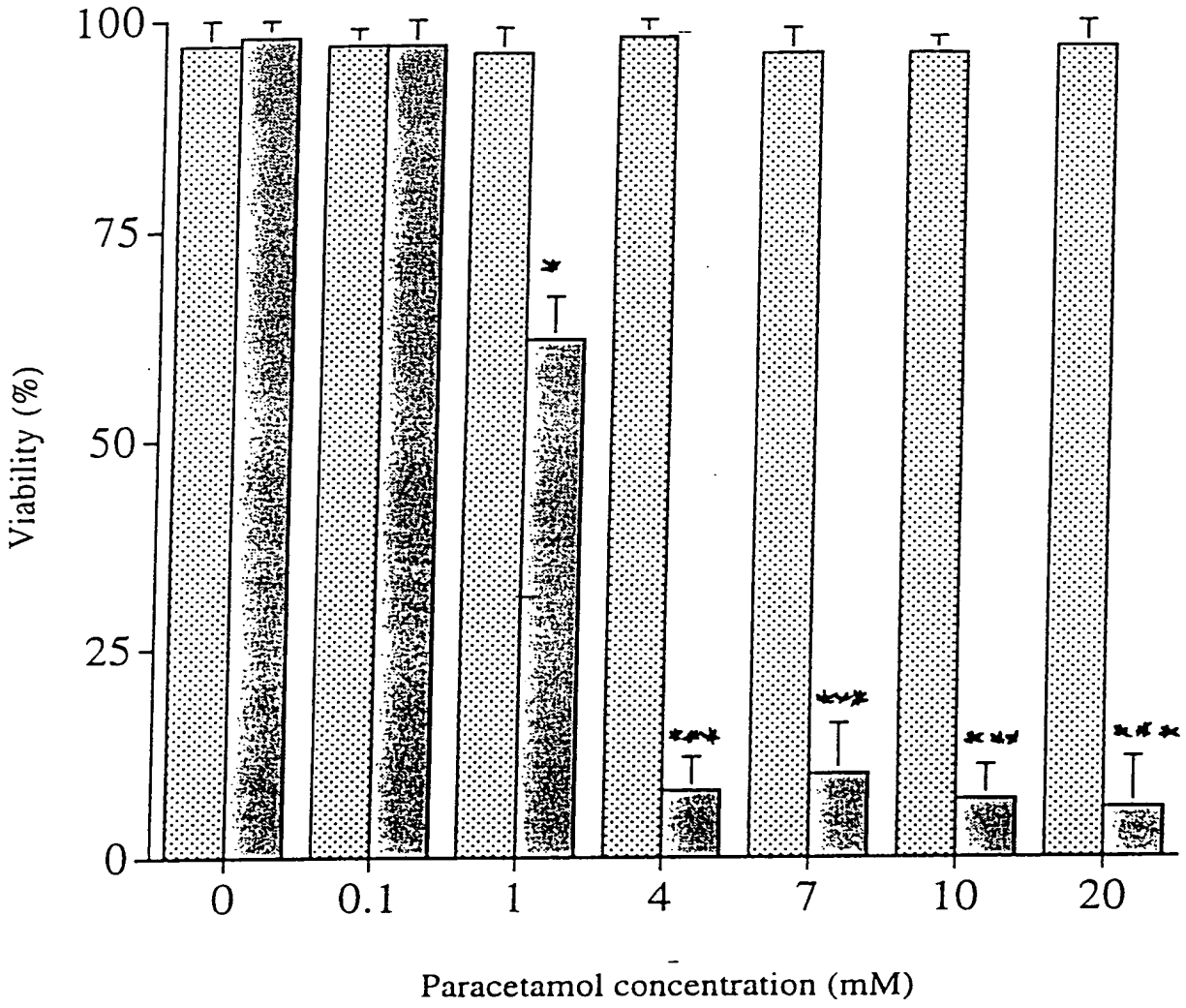
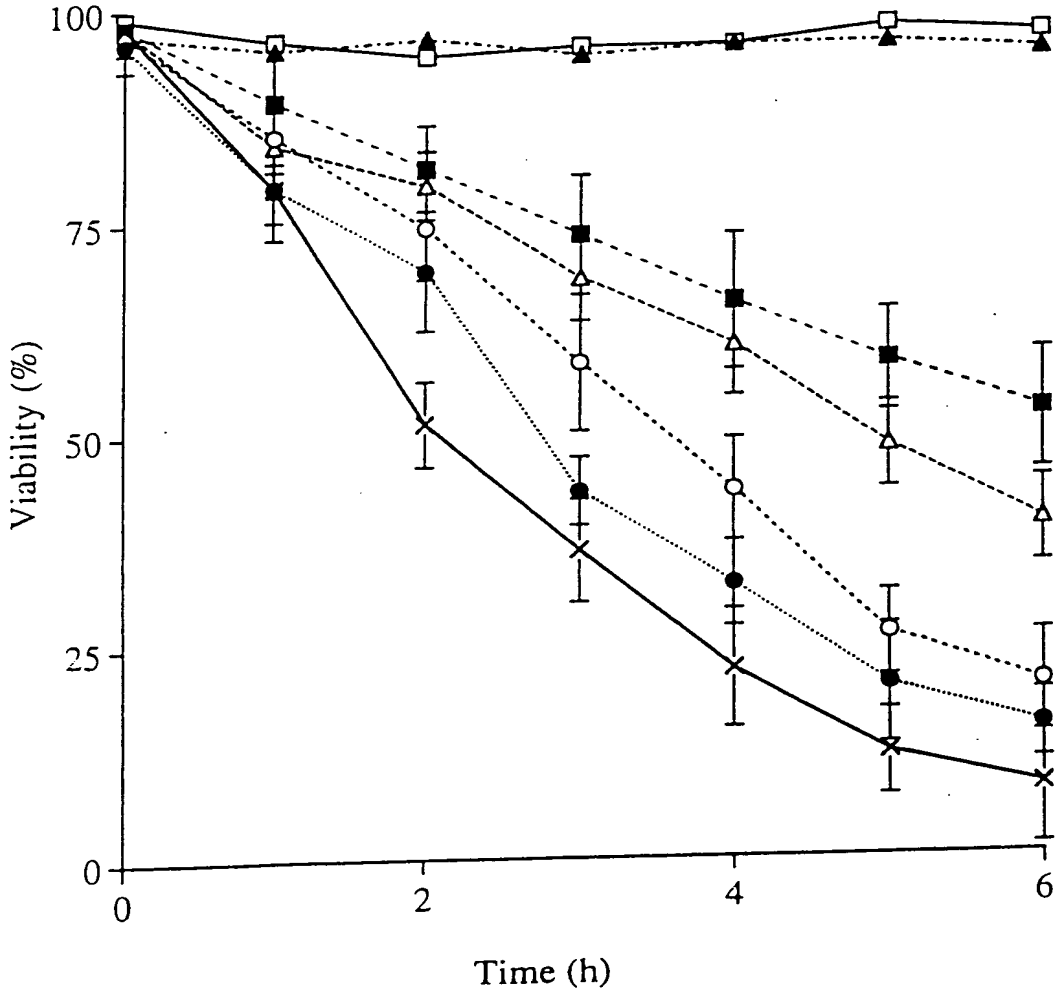

100
75
50
25
0
Viability (%)
0
0.1
1
4
7
10
20
Paracetamol concentration (mM)

Fig. 2



PCT/GB[redacted]4268

28/1/00 CP

Harrison Goddard Foote